SYNOPSIS

Japanese encephalitis virus (JEV), a member of the genus Flavivirus in the family *Flaviviridae* is recognized as the leading cause of childhood viral encephalitis in Asia with approximately 50,000 cases and 10,000 to 15,000 deaths reported annually. Approximately 50% of the survivors display long-term neurological impairments that manifest as motor paralysis, speech and movement disorders, mental deficiency and abnormal emotional behavior. JE is endemic in many parts of Asia, including India, Nepal, Srilanka, Burma, China, Indonesia, Japan, Korea, Malaysia, Thailand, and Vietnam. Children one to fifteen years of age are predominantly affected in endemic areas while adult infections occur in areas where the disease is newly introduced. With no specific antiviral drugs available for JEV at present, vaccination is the only effective approach to prevent JE. A highly purified, formalin inactivated mouse brain derived commercial JE vaccine has been the most widely used and until recently, has been the only JE vaccine licensed in the international market. Although the efficacy of this vaccine is 91%, it has several limitations including very high manufacturing cost and high risk of exposure to extremely neurovirulent virus during manufacturing, in addition to stimulating systemic and allergic reactions in the vaccinees, attributed to mouse brain derived components in this preparation. Most importantly, for effective immunization the inactivated vaccine requires multiple doses because of its failure to maintain long-term memory. All these factors increase the cost of the vaccine, making it impractical to implement a common vaccination program in developing countries like India. Although an effective live attenuated SA 14-14-2 JE vaccine has been in use in China since 1988,

this vaccine is being introduced into other affected Asian countries including Nepal, South Korea and India only recently.

For protection against JEV, both neutralizing antibodies and virus specific T cells, including cytotoxic T lymphocytes (CTLs) have been considered important. The fact that majority of JEV-exposed individuals clear the viral infection without displaying the disease symptoms and that adults in JE-endemic areas rarely come down with disease suggest that repeated exposure to the virus in JE-endemic areas results in the development of protective immune responses. Identification of the JEV proteins targeted by these protective immune parameters will provide valuable information for the development of an improved JE vaccine. JEV is an enveloped virus with single strand, positive sense RNA genome, which encodes a single polyprotein that is co- and post-translationally cleaved to produce three structural proteins- capsid (C), membrane (M)/ precursor membrane (prM), Envelope (E) and seven non-structural (NS) proteins - NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5.

We investigated the immune responses prevalent in convalescent JE patients and JEV exposed healthy individuals from Karnataka and Andhra Pradesh states of India. Our previous studies in individuals residing in JEV-endemic regions revealed T cell immunodominance of non structural proteins NS3 and NS1 during natural JEV infections in humans where as the structural protein E, which is a good target for neutralizing antibody response and the major component of the commercially available formalin inactivated vaccine is a poor inducer of T cells.

Role of JEV NS1 in protective immunity and in immunopathology. Flavivirus NS1 exists as intracellular, cell surface associated, and extracellular non-virion forms. NS1 is also known to induce humoral immune response. Several studies in different flaviviruses have indicated a role for NS1-specific immune responses in protection against flaviviruses. Passive immunization studies in mice using monoclonal antibodies (MAbs) to Yellow fever virus (YFV), West Nile virus (WNV), and dengue virus (DENV) NS1 showed that these antibodies are protective. Passive protection mediated by MAb to YFV NS1 correlated with *in vitro* complement mediated cytolysis of YFV infected cells. Similarly, many studies also showed that active immunization with purified NS1 protein, plasmid DNA or recombinant vaccinia virus expressing NS1 provides protection against lethal viral challenge. Paradoxically, studies also pointed to the contribution of NS1 in pathology and immune modulation. Antibodies directed to DENV NS1 were shown to cross react with platelets and extracellular matrix proteins as well as endothelial cells and cause inflammation and apoptosis. Immunization with DENV NS1 led to immune mediated liver injury in mice (Lin, C-F. *et al*., 2008. Lab Invest 88: 1079-1089). Recently, binding of soluble DENV NS1 to uninfected cells and tissues have been demonstrated and was implicated in the vascular leakage syndrome in severe DENV infections (Avirutnan, P. *et al*., 2007. PLoS Pathog 3: e183). Moreover, serum NS1 levels in DENV and WNV infected patients directly correlated with disease severity. Ability of WNV NS1 to bind to human complement regulatory protein factor H suggested a role of NS1 in immune modulation (Chung, K.M. *et al*., 2006. Proc Natl Acad Sci USA 103: 19111-19116). Although literature is available on the contribution of immune responses to JEV NS1 towards protection in animal models, its role in human JEV infections and its

contribution to immunopathology or immune modulation in humans or animal models is not reported. We screened serum samples from 72 convalescent JE patients for the presence of anti-NS1 antibodies by ELISA and radioimmunoprecipitation and found NS1 reactivity in 45 samples. First we wanted to address the question whether these antibodies to NS1 present in the serum of JEV infected human volunteers are capable of inducing complement mediated cytolysis of cells expressing NS1 on the surface. Since these human serum samples also contain antibodies to E and since JEV infected cells express both E and NS1 on the surface, we used a recombinant vaccinia virus to express JEV NS1 alone on the surface of target cells without any other JEV proteins. We generated recombinant vaccinia virus expressing JEV NS1 with its signal sequence (vNS1ss) and observed that the recombinant NS1 expressed by vaccinia virus is identical to authentic JEV NS1 in forming DTT stable dimers, expression on the surface of infected cells and secretion into the culture medium. Our results using BHK-21 cells or human derived SW-13 cells expressing JEV NS1 on the surface showed significant cytolysis by anti-NS1 antibody containing human serum in the presence of rabbit complement (with BHK-21 cells) or human complement (with SW-13 cells). Additionally, we demonstrated twenty two fold reduction in the infectious virus produced at 48h in SW-13 cells in the presence of human complement and NS1 antiserum compared to control serum, suggesting that complement mediated cytolytic activity of anti-NS1 antibody helps the host in controlling the virus propagation. Interestingly, antibodies to JEV NS1 did not bring about cross cytolysis of West Nile virus (WNV) or dengue virus (DENV) infected cells despite cross recognition of their NS1 proteins. The role of JEV NS1 in immunopathology and immune modulation was next addressed. We used culture supernatant of JEV and vNS1ss infected

cells enriched for NS1 by ammonium sulphate fractionation for this purpose. Our results showed that JEV NS1, unlike DENV NS1 was not capable of binding to the surface of Vero, HepG2 and BHK-21 cells. Moreover, JEV NS1 failed to bind to complement regulatory protein factor H, a property which was demonstrated for WNV NS1 and was implicated in immune modulation by decreasing complement recognition of infected cells. In vivo experiments in the mouse model showed that the pre-existing circulating antibodies to JEV NS1 do not enhance severity of subsequent DENV or WNV infection. In fact, these mice were protected from lethal WNV challenge. Taken together these results suggest that JEV NS1 contributes to protective immune response without causing immunopathology or immune modulation. This is further substantiated by the observation of direct correlation of presence of anti-NS1 antibodies in the JE convalescent patients' serum with better prognosis. We concluded from these findings that inclusion of NS1 in a vaccine should therefore enhance vaccine efficacy (Krishna, V.D. *et al*., 2009. J Virol 83: 4766-4777).

Comparison of immune responses to JEV structural proteins Capsid and Envelope in human volunteers vaccinated with inactivated JE vaccine and naturally exposed to live JEV. Formalin inactivated JE vaccine contains only structural proteins of JEV and the non structural proteins are absent in this preparation. As mentioned earlier, inactivated JE vaccine is a poor inducer of long term immunity. We hypothesized that the absence of dominant T cell antigens NS3 and NS1 in the inactivated virus preparation could be one of the reasons for its inability to induce long term memory immune response since T cell help is required for stimulating long lasting immunity. We first

enquired whether differences in CMI responses to JEV structural proteins might be observed when provided as inactivated virus preparation without competing non structural proteins compared to that elicited upon live virus infection. We therefore measured CMI responses elicited by two of the three structural proteins C and E in human volunteers vaccinated with commercially available inactivated JE vaccine (n = 13) and in healthy, live JEV exposed individuals recruited from JE-endemic regions (n = 23). We found that the magnitude of lymphoproliferation in response to E was higher in live JEV exposed individuals compared to vaccinees while the level of IFN-γ produced in response to both C and E were similarly low in the two groups of volunteers. Both $CD4^+$ and $CD8^+$ T cells produced IFN-γ in live JEV exposed individuals where as IFN-γ was predominantly produced by $CD4^+$ cells in killed JE vaccine immunized individuals. Taken together, these results suggest that structural proteins E and C are inherently poor inducers of T cells even in killed vaccine preparation, where there is no competition from immunodominant non structural proteins. In conclusion, inclusion of nonstructural proteins NS1 and NS3 along with neutralizing antibody inducing E should improve memory and consequent efficacy of the JE vaccine.

Construction and testing in the Mouse Model of experimental recombinant poxvirus vaccines Expressing prM, E, NS1, and NS3 of JEV. Guided by the information on immune responses to JEV in the JE endemic human cohort and volunteers vaccinated with killed JE vaccine, we designed experimental vaccines as recombinant vaccinia viruses expressing NS1, NS3, prM, and E proteins of JEV (vNS1NS3prME) or NS1, NS3, prM, and C-terminally truncated E (vNS1NS3prMΔE), since this form of E was

reported to induce greater neutralizing antibody response and higher levels of protective immunity in mice compared to the full length E protein (Jan, L.R. *et al*., 1993. Am J Trop Med Hyg 48: 412-423). We also constructed recombinant vaccinia virus expressing only non structural proteins NS1 and NS3 (vNS1NS3) in addition to NS1 alone (vNS1ss). However, multiple attempts to express prM along with E or ΔE in recombinant vaccinia virus were not successful. We studied the immune responses elicited by these experimental vaccines in mice after immunization. Our data showed that a recombinant vaccinia virus expressing prM, ΔE, NS1, and NS3 of JEV (vNS1NS3prMΔE) is superior to formalin inactivated JE vaccine (JEvac) in eliciting long lived neutralizing antibodies. In addition, immunization with recombinant vaccinia viruses elicited NS1-specific antibodies with the ability to induce complement mediated lysis.

We next analyzed the antigen specific proliferative response and IFN-γ production in response to purified E, NS1, and NS3 in the inguinal lymph node cells and splenocytes of immunized mice. As expected JEvac immunized mice responded only to E and not to nonstructural proteins tested. Moreover, both magnitude of lympho proliferation and level of IFN-γ production in response to E were significantly lower in JEvac immunized mice compared to vNS1NS3prMΔE immunized mice. Flowcytometry analysis of IFN-γ producing cells showed that lymphocytes from JEvac immunised mice predominantly recruited $CD4^+$ over $CD8^+$ T cells when stimulated *in vitro* with purified E whereas vNS1NS3prMΔE recruited both $CD4^+$ and $CD8^+$ T cells to produce IFN-γ similar to that observed in lymphocytes from live JEV immunized mice. Furthermore, we demonstrated the ability of JEV specific CTLs to recognize WNV infected target cells only from live JEV or recombinant vaccinia virus immunized mice but not from JEvac

immunized mice, suggesting that these cross reactive CTLs are specific to NS1 or NS3. Finally, to evaluate the protective immunity in recombinant vaccinia virus immunized mice we challenged these mice with 125 LD_{50} of JEV six weeks after the final boost. vNS1NS3prMΔE provided the maximum protection with 85.71% survival, statistically similar to mice immunized with JEvac that provided 62.5% protection. However, challenge studies performed six months after the final booster dose revealed significantly lower protection in JEvac immunized mice (37.5% survival) compared to vNS1NS3prMΔE immunized mice (87.5% survival). Importantly, when challenged 1 year after administering the final booster dose none of the JEvac immunized mice survived after lethal JEV challenge (0/4) whereas vNS1NS3prMΔE provided 83.33% protection (5/6). Thus, inclusion of non structural proteins together with the live nature of the vNS1NS3prMΔE makes this vaccine superior to inactivated JE vaccine in eliciting long lived neutralizing antibodies as well as NS1 and NS3-specific CTLs along with NS1-specific antibodies with the ability to induce complement mediated lysis, resulting in long lasting and enhanced protection from lethal JEV infection in mice.

Our results thus identified both B and T cell antigens whose inclusion in a live-vectored vaccine would provide long lasting immunity with far superior efficacy over the inactivated JE vaccine.